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Functional genomics provides insights into the role of *Propionibacterium freudenreichii* ssp. *shermanii* JS in cheese ripening

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ABSTRACT

Propionibacterium freudenreichii is a commercially important bacterium that is essential for the development of the characteristic eyes and flavor of Swiss-type cheeses. These bacteria grow actively and produce large quantities of flavor compounds during cheese ripening at warm temperatures but also appear to contribute to the aroma development during the subsequent cold storage of cheese. Here, we advance our understanding of the role of *P. freudenreichii* in cheese ripening by presenting the 2.68-Mbp annotated genome sequence of *P. freudenreichii* ssp. *shermanii* JS and determining its global transcriptional profiles during industrial cheese-making using transcriptome sequencing. The annotation of the genome identified a total of 2377 protein-coding genes and revealed the presence of enzymes and pathways for formation of several flavor compounds. Based on transcriptome profiling, the expression of 348 protein-coding genes was altered between the warm and cold room ripening of cheese. Several propionate, acetate, and diacetyl/acetoin production related genes had higher expression levels in the warm room, whereas a general slowing down of the metabolism and an activation of mobile genetic elements was seen in the cold room. A few ripening-related and amino acid catabolism involved genes were induced or remained active in cold room, indicating that strain JS contributes to the aroma development also during cold room ripening. In addition, we performed a comparative genomic analysis of strain JS and 29 other *Propionibacterium* strains of 10 different species, including an isolate of both *P. freudenreichii* subspecies *freudenreichii* and *shermanii*. Ortholog grouping of the predicted protein sequences revealed that close to 86% of the ortholog groups of strain JS, including a variety of ripening-related ortholog groups, were conserved across the *P. freudenreichii* isolates. Taken together, this study contributes to the understanding of the genomic basis of *P. freudenreichii* and sheds light on its activities during cheese ripening.

1. Introduction

The genus *Propionibacterium* belongs to the high GC% phylum of Gram-positive bacteria (Actinobacteria) (Goodfellow, 2012) and its members have a distinguishable ability to produce large quantities of propionate and acetate (Patrick and McDowell, 2012). They have traditionally been classified into cutaneous and dairy groups based on their natural habitats, although this division reflects poorly on the 16S rRNA-based phylogeny of the genus (Patrick and McDowell, 2012). The cutaneous group mainly comprises species that are present in the vertebrate skin and gastrointestinal tract and includes the opportunistic pathogen *Propionibacterium acnes*, presumably having a role in the formation of acne (Dessinioti and Katsambas, 2010). Members of the dairy group inhabit primarily dairy and silage environments, and many of them have a long history of safe use in food manufacturing (Poonam et al., 2012). This group also includes *Propionibacterium freudenreichii* that is used in the commercial production of B-vitamins and is a known bio-preservative (Le Marechal et al., 2015; Poonam et al., 2012). The species is also essential in the manufacturing of Swiss-type cheeses. The eyes typical for this cheese type result from extensive formation of CO_2 during the growth of *P. freudenreichii*, while the aroma compounds it produces through lactate fermentation, amino acid catabolism, and milk fat hydrolysis contribute markedly to the development of the characteristic flavor (Poonam et al., 2012; Thierry et al., 2011). Moreover, some *P. freudenreichii* strains are of interest because of their potential to modulate intestinal function and normal flora (Cousin et al., 2012; Thierry et al., 2011).

Abbreviations: Cas, CRISPR-associated protein; CDS, coding sequence; COG, clusters of orthologous groups; CRISPR, clustered regularly interspaced short palindromic repeats; EMPP, Embden-Meyerhof-Parnas-pathway; FDR, false discovery rate; GI, genomic island; HGT, horizontal gene transfer; LAB, lactic acid bacteria; TCA, tricarboxylic acid cycle; TU, transcription unit.

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Currently, over a hundred complete or scaffold-level *Propionibacterium* genome entries are available in GenBank (July 2016). These genome sequences are on average ~2.58 Mb in size and have an average GC content of ~62%. A majority of them represent *P. acnes*, while 21 entries describe *P. freudenreichii* strains. These sequences have advanced our knowledge of the role of propionibacteria in human health and disease and shed light on the genomic basis of commercially important features. In particular, a recent comparative genome analysis of 82 *P. acnes* isolates revealed genetic differences that may underlie the different roles of *P. acnes* as a commensal and pathogen (Tomida et al., 2013). The genome analyses of *P. freudenreichii* have, in turn, described metabolic pathways for the production of cheese flavor compounds in *P. freudenreichii* ssp. *shermanii* CIRM-BIA1 (Falentin et al., 2010a) and provided foundations to identify host-interaction and health-enhancing factors in *P. freudenreichii* ssp. *freudenreichii* ITG P20 (Le Marechal et al., 2015). Genomic comparison of strain *P. freudenreichii* ssp. *shermanii* CIRM-BIA1 with *P. acnes* KPA171202 has also revealed that most virulence-related genes of strain KPA171202 are absent in strain CIRM-BIA1 (Falentin et al., 2010a), a finding that supports the safe status of *P. freudenreichii*.

The current genome information of *P. freudenreichii* is complemented by a few gene expression studies that have primarily focused on the function of this bacterium in cheese ripening. For instance, quantification of the expression levels of selected genes in microbiologically controlled cheese samples has revealed *P. freudenreichii* to be metabolically most active at the end of the initial cheese ripening in cold room (+12 °C) and to retain metabolic activity during the first two weeks in subsequent warm room ripening (+24 °C) (Falentin et al., 2010b). Microarray-based global gene expression profiling of *P. freudenreichii* in laboratory settings mimicking cheese ripening and cold storage has suggested that *P. freudenreichii* is metabolically active and continues to produce flavor compounds during cold storage (+4 °C), although in lesser extent than in warm conditions (+30 °C) (Dalmasso et al., 2012). In addition to the ability to adjust to the different cheese manufacturing conditions, microarray-based gene expression profiling has been used in investigating *P. freudenreichii* transcriptome responses to conditions encountered in the gut (Saraoui et al., 2013).

In the present study, we broaden the understanding of dairy propionibacteria and *P. freudenreichii* by describing the complete and annotated genome sequence of *P. freudenreichii* ssp. *shermanii* JS. Special emphasis was placed on the genes associated with flavor-production and other factors relevant in the dairy environment. To elucidate the conservation level of the 2377 protein-coding genes of strain JS, a comparative analysis of the gene contents of the strain JS and 29 other *Propionibacterium* strains was performed. This analysis indicated that ~91% of the orthology groups of strain JS had an orthologous counterpart in at least one of the two other *P. freudenreichii* genomes publicly available at the time of the analysis. In addition, the gene expression responses of strain JS to cheese ripening stages was determined. As the gene transcription of *P. freudenreichii* during cheese ripening has previously been studied only in setups including a very small subset of genes (Falentin et al., 2010b) or in genome-wide setups in laboratory conditions (Dalmasso et al., 2012), we focused on the gene expression of this bacterium in industrial cheese manufacturing. Using RNA-sequencing (RNA-seq), the expression of 348 protein-coding genes of strain JS were found to differ ≥2-fold with false discovery rate (FDR) of ≤0.05 between the warm (+20 °C) and cold (+5 °C) ripening of cheese. Of these differentially expressed genes, 128 were up-regulated in the warm room ripening, including various genes playing a role in cheese flavor and structure development. In contrast, various functionally unclassified genes and mobile genetic elements, as well as a few ripening-related genes, were induced during the cold room ripening. A general suppression of carbon metabolism in the cold room was also evident from the data, indicating that *P. freudenreichii* grew and contributed to the flavor development more during the warm room ripening of cheese.

2. Materials and methods

2.1. Culture conditions, extraction of genomic DNA, and genome sequencing

P. freudenreichii ssp. *shermanii* JS was obtained from the Valio culture collection (Valio Ltd). Cells were cultivated anaerobically at +30° C for 17 h in propioni medium (pH 6.8) consisting of 0.5% (w/v) tryptone (LabM), 1% (w/v) yeast extract (LabM), 1.0% (w/v) sodium lactate (Merck) and supplemented with 1% glycine (Merck). Genomic DNA for genome sequencing was extracted using a method modified from (Anderson and McKay, 1983) and the complete genome sequence of strain JS was determined using a combination of different sequencing and gap-closure approaches and assembly algorithms (Supplemental text). A combination of approaches was applied to annotate and analyze the genomic data of strain JS as described in Supplemental text. The annotated genome sequence is accessible through the European Nucleotide Archive (PRJEB12148; http://www.ebi.ac.uk/ena/data/view/PRJEB12148).

2.2. Comparative genomics and phylogenetic analysis

Ortholog groups among strain JS and 29 other *Propionibacterium* isolates with publicly available genome sequences in December 2014 (Table S1) were identified as described in Supplemental text. Phylogenetic relationships between strains were computed based on 486 orthologous protein sequences present as a single copy in each strain. In addition, whole-genome comparative analysis of *P. freudenreichii* ssp. *shermanii* strains JS and CIRM-BIA1 (Falentin et al., 2010a), *P. freudenreichii* ssp. *freudenreichii* ITG P20 (Le Marechal et al., 2015), and *P. acnes* KPA171202 (Bruggemann et al., 2004) was performed to reveal genome-level similarities and differences between these strains. More information on the comparative and phylogenetic analyses is available in Supplemental text.

2.3. Cheese sampling

DNA for amplicon sequencing and RNA for transcriptome profiling were extracted from three matched warm and cold room cheese samples during ripening (Fig. 1). Semi hard Maasdam-type cheese (moisture 51.1%, fat 15%, and salt 1.5%) was cooked with mesophilic cooking recipe and with mesophilic starter cultures *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris* and *Lactobacillus rhamnosus*, in combination with thermophilic *Lactobacillus helveticus* and *P. freudenreichii* ssp. *shermanii* JS. After cooking, cheeses were moulded and pressed. After brining, three 13 kg cheeses (A, B, C) from the same cooking batch were cut into four parts that were packaged individually in plastic ripening foil. The cheeses were ripened in warm room (+20 °C) for 30 days, after which they were transferred into cold room (+5 °C). One quarter of each of the three cheeses was sampled at day 12 (*i.e.* warm room samples A1, B1, and C1) and another quarter at day 37 of ripening (*i.e.* cold room samples A3, B3, and C3) (Fig. 1). The outer rims of the quarters were cut off and only the central parts were used for nucleic acid extractions.

2.4. Amplicon sequencing and 16S rRNA gene analysis

Genomic DNA for amplicon sequencing was extracted from cells isolated from 10 g of cheese, and the V1–V3 region of 16S rRNA gene was amplified, sequenced, and analyzed as described in Supplemental text. The amplicon sequence data is accessible through Sequence Read Archive (Bioproject PRJNA317284; http://www.ncbi.nlm.nih.gov/sra/).

2.5. RNA-sequencing and transcriptome analysis

RNA extracted from cells isolated from 10 g of cheese was prepared and sequenced as described in Supplemental text. Generated reads

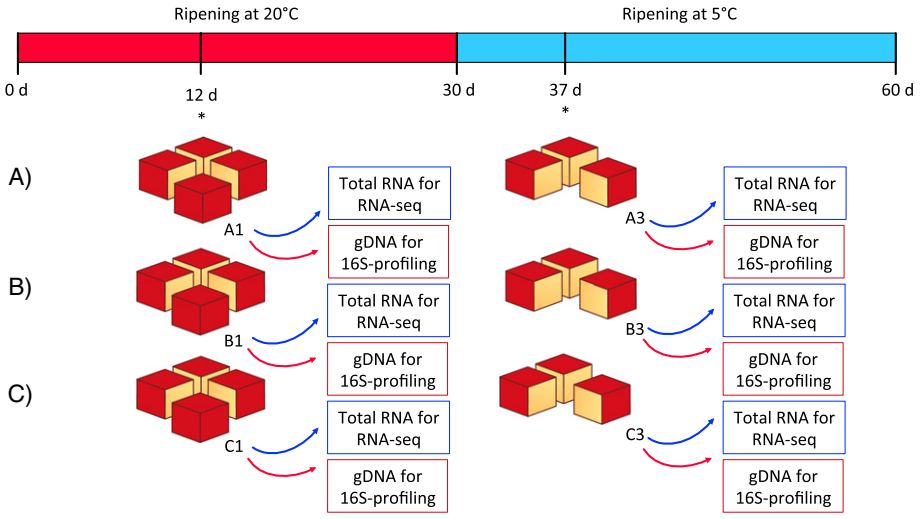

Fig. 1. Cheese sampling. Three cheeses (A, B, C) were produced from the same cooking batch and divided into individually packaged quarters for ripening. One quarter of each cheese was sampled (*) on the 12th day of ripening (warm room samples: A1, B1, C1) and one on the 37th day of ripening (cold room samples: A3, B3, C3). Total RNA and DNA were extracted from each sample for RNA-seq-based transcriptome profiling and 16S rRNA gene-based community profiling, respectively.

were mapped onto the genome of strain JS and used for differential gene expression analysis (Supplemental text). Genes with FDR ≤0.05 and fold-change ≥2-fold were considered as differentially expressed. The statistical significance of the enrichment and depletion of up- and down-regulated genes over all JS genes in KEGG pathways and clusters of orthologous groups (COG) categories was also tested and sequence motifs over-represented in upstream regions of cold room- and warm room-induced transcription units (TUs) searched (Supplemental text). RNA-seq data are accessible in the ArrayExpress database (E-MTAB-4593; http://www.ebi.ac.uk/arrayexpress).

3. Results and discussion

3.1. General genomic features

The genome of *P. freudenreichii* ssp. *shermanii* JS was sequenced using a combination of different sequencing technologies to approximately 691× coverage and annotated. The final assembly of the complete genome sequence of strain JS consisted a single circular chromosome of 2,675,045 bp in size with an overall GC content of 67.23% and was devoid of plasmids. The genome was identified to contain sequence motifs with a 6-methyladenine base modifications (Table S2) that are potentially involved in restriction modification systems (discussed in Supplemental text) and was predicted to encode two rRNA clusters, 45 tRNA-genes, and 2377 coding sequences (CDSs). Putative biological functions were assigned to ~88% of the CDSs by at least one of the functional annotation tools, while ~7% were classified as conserved putative proteins and ~5% as having no match to known proteins. The genome was also predicted to encode a complete CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR-associated proteins) system for defense against phages and other invading nucleic acids (Table 1; Supplemental text). The general genomic characteristics and the annotation results of strain JS are summarized in Table 1 alongside with those of previously characterized *P. freudenreichii* cheese starters CIRM-BIA1 and ITG P20 (Falentin et al., 2010a; Le Marechal et al., 2015) and that of *P. acnes* KPA171202, which was the first *Propionibacterium* having its genome published (Bruggemann et al., 2004).

3.2. Comparative genomics with other propionibacteria

To elucidate the distribution of the proteinaceous features of strain JS across propionibacteria, CDS translations of strain JS and 29 other propionibacteria representing 10 different species (Table S1) were assigned to ortholog groups. Using OrthoMCL (Li et al., 2003), we were able to classify the 72,283 CDSs possessed by this selection of strains into 10,091 ortholog groups (*i.e.* pan-genome; Supplemental file 1). Of these ortholog groups, 4154 (~41%) were present in only a single strain, 1909 (~19%) were conserved across the three *P. freudenreichii* isolates, and 546 (~5%) were common to all of the *Propionibacterium* strains (*i.e.* core genome) (Fig. 2). The ~5% proportion of common groups compared with the total number of groups was similar to that seen in the analysis of 14 *Bifidobacterium* genomes representing 9 species (~10%) (Bottacini et al., 2010), which is another genus within the class *Actinobacteria* and well characterized at the genome-level. In contrast, previous analyses (Parizzi et al., 2012) focusing on three *Propionibacterium* strains of three different species and 21 recently characterized *P. freudenreichii* strains (Loux et al., 2015) have suggested *Propionibacterium* and *P. freudenreichii* strains to share 1026 (~26%) and 1343 (~12%) of their ortholog groups, respectively. These differences are best explained by the different numbers of genomes included in the analyses, as the number of common groups generally decreases with the addition of genome sequences (Medini et al., 2005).

The groups conserved across all 30 *Propionibacterium* strains represented nearly 25% of the ortholog groups of strain JS, whereas only ~7% of the ortholog groups of this strain were strain-specific (Fig. 2). These strain-specific ortholog groups comprised altogether 178 CDSs in strain JS (Supplemental file 1) and formed ~7% of the genetic complement of this strain. This portion of strain-specific genes is slightly more

Table 1
General genomic features of selected *Propionibacterium* strains.

	JS	CIRM-BIA1	ITG P20	KPA171202
Scaffolds	1	1	59	1
Genome size (Mb)	2.68	2.62	2.59	2.56
GC%	67.23	67.30	67.20	60.00
tRNA	45	45	45	47
rRNA clusters	2	2	[a]	3
CDSs	2377	2326	2307	2297
Ortholog groups	2232	2224	2268	2265
CRISPR/Cas system	Type I	Type I	NA	NA
Prophage clusters	1	NA	NA	1
GIs (CDSs/strain-specific CDSs)	11 (181/29)	14 (198/43)	9 (89/28)	6 (71/10)
Coverage over the JS genome (%)	100	94	92	29
Identity against the JS genome (%)	100	99	99	84

[a] Only partial rRNA cluster.

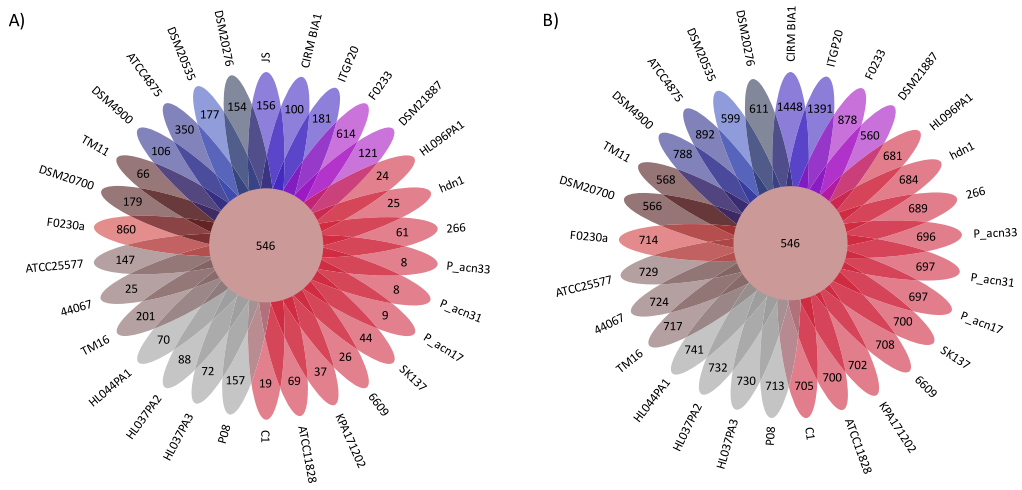

Fig. 2. Strain-specific ortholog groups and ortholog groups conserved between strain JS and 29 other *Propionibacterium* strains. Petals in panel A depict the number of strain-specific ortholog groups for each of the *Propionibacterium* strains analyzed and petals in panel B indicate the number of non-core ortholog groups shared between a given strain and strain JS. Petals representing members of the same species are colored in the same color. The number of ortholog groups conserved across all 30 propionibacteria is given in the center of both flower-plots.

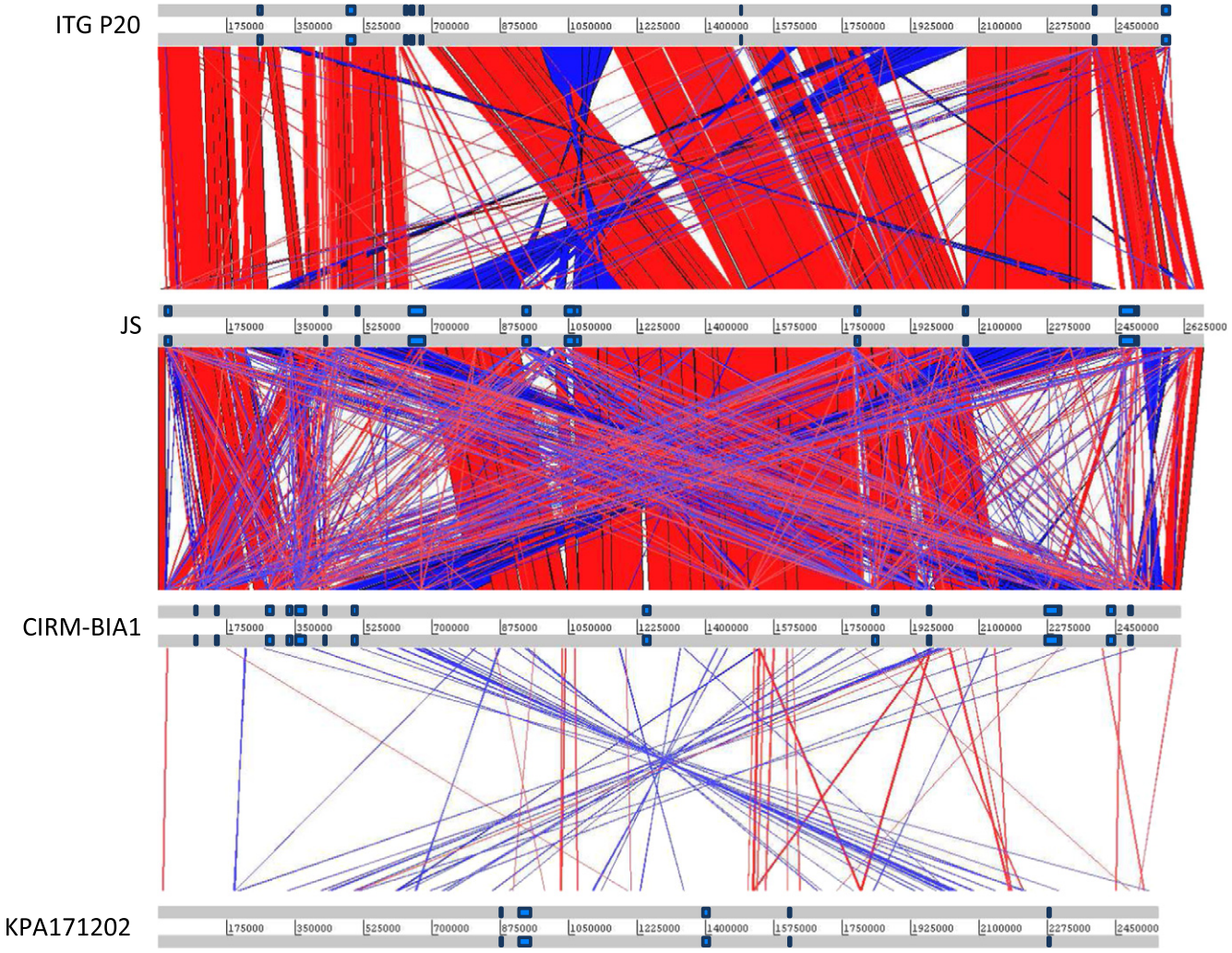

Fig. 3. Whole-genome comparison of cheese ripening-associated *P. freudenreichii* strains and a representative *P. acnes* strain. Strains JS and CIRM-BIA1 belong to *P. freudenreichii* ssp. *shermanii*, strain ITG P20 to *P. freudenreichii* ssp. *freudenreichii*, and KPA171202 to *P. acnes*. Contigs of strain ITG P20 draft genome were ordered to reflect genome architecture of strain JS. Vertical bands represent significant sequence matches. Blue boxes on the genome strands represent GIs.

than that of strain CIRM-BIA1 (~4%) and slightly less than that of strain ITG P20 (~8%) and that previously reported for other *P. freudenreichii* strains (Loux et al., 2015). As expected, strain JS shared most of its ortholog groups with the other *P. freudenreichii* strains CIRM-BIA1 (~89%) and ITG P20 (~87%) (Fig. 2B). These shared groups included, for instance, several flavor-related genes and genes associated with the central carbon metabolism and amino acid biosynthesis, which are discussed in more detail in the next chapter.

A detailed whole-genome comparison of *P. acnes* KPA171202 and *P. freudenreichii* strains JS, CIRM-BIA1, and ITG P20 revealed low nucleotide and synteny similarity between *P. freudenreichii* and *P. acnes* (Fig. 3 and Table 1), which is in accordance with previous observations (Falentin et al., 2010a) and ortholog analysis (Fig. 2B). Genomes of the three analyzed *P. freudenreichii* strains shared a notable nucleotide similarity and coverage (Table 1). Genomic rearrangements between the different *P. freudenreichii* strains were, however, detected (Fig. 3). Whether these rearrangements are genuine or a consequence of different sequencing approaches and reads of varying insert and read lengths cannot be unambiguously inferred from the data. Interestingly, each *P. freudenreichii* genome contained several unconserved genome regions that co-localized with or were found in the close vicinity of putative genomic islands (GIs) (Fig. 3), indicating that they may have been the results of recent horizontal gene transfer (HGT) events. These GIs covered a larger portion of the genome of strain JS (205 kb) than of those of strains CIRM-BIA1 (~184 kb) and ITG P20 (~72 kb) (Table 1) and contained several genes without orthologous counterparts in any of the 29 other *Propionibacterium* strains included in the ortholog grouping. For example, out of the 181 CDSs located in the GIs of strain JS, 39 were specific for strain JS (Table 1). By contrast, one of the identified GIs in the JS genome (*PFREUDJS_002184-PFREUDJS_002217*) harbored genes for the utilization of lactose, a property, that was recently reported to be conferred by a GI in *P. freudenreichii* (Loux et al., 2015). Similar to the previously identified GI-encoded lactose utilization loci (Loux et al., 2015), the lactose utilization loci in strain JS was also surrounded by transposases.

To examine the phylogenetic relations between *Propionibacterium* strains, we built a maximum-likelihood tree from the multiple sequence alignment of 486 orthologous proteins present as a single copy in each of strain. The phylogeny (Figs. 4 and S1) showed that strains of the same species clustered together and revealed a branching order that was similar to that reported in previous studies (Lucena-Padros et al., 2014; Patrick and McDowell, 2012). The phylogenetic tree also confirmed the positioning of strain JS within *P. freudenreichii*.

3.3. Metabolic pathways and metabolism

During cheese ripening, *P. freudenreichii* ferments lactate excreted by lactic acid bacteria (LAB) into the flavor-forming propionate and acetate as well as into CO_2 that is responsible for the eye formation in cheese. As expected, the *in silico* reconstruction of the *P. freudenreichii* ssp. *shermanii* JS metabolic pathways revealed several pathways that are central in cheese fermentation and lead to the formation of flavor compounds (summarized in Fig. 5). Similarly to strain CIRM-BIA1 (Falentin et al., 2010a), strain JS possessed genes for enzymes responsible for the conversion of lactate into pyruvate and forming the two key metabolic cycles involved in propionate production, namely, the Wood-Werkman cycle and the citric acid or tricarboxylic acid (TCA) cycle (Fig. 5). Similarly, strain JS encoded the enzymatic machinery for the production of the flavor compound acetate as well as Embden-Meyerhof-Parnas-pathway (EMPP) for hexose utilization (Fig. 5).

Interestingly, strain JS also seemed to have the ability to produce buttery note excreting diacetyl and acetoin from pyruvate (Fig. 5). The production of these flavor compounds is known to occur by some propionibacteria (Hettinga and Reinbold, 1972), and the diacetyl and acetoin production related genes (*PFREUDJS_001285*, *PFREUDJS_001286*, and *PFREUDJS_001113*) were present also in some other of the 29 propionibacteria analyzed, including *P. freudenreichii* strains CIRM-BIA1 and ITG P20 (Supplemental file 1). The predicted steps involved in the conversion of pyruvate to diacetyl and acetoin are similar to those of the diacetyl/acetoin producing citrate positive lactococci and *Leuconostoc* (McSweeney and Fox, 2004). Briefly, pyruvate is first converted into acetolactate, which can be enzymatically converted into acetoin or non-enzymatically into diacetyl (Fig. 5). However, no diacetyl reductase- (EC 1.1.1.303) or butanediol dehydrogenase (EC 1.1.1.4) -encoding genes were detected in the JS genome, suggesting that strain JS is unable to convert diacetyl to acetoin or reduce acetoin to 2,3-

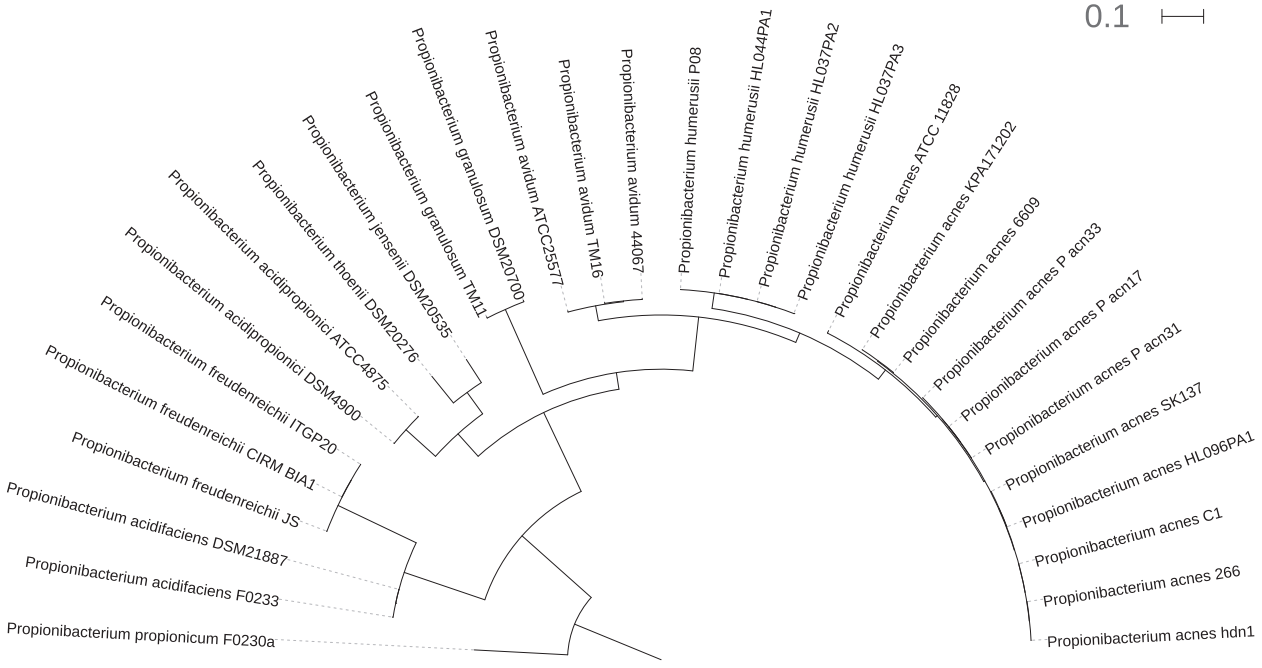

Fig. 4. Phylogenetic tree of 30 *Propionibacterium* strains. The phylogenetic tree was constructed based on the 486 orthologous proteins present as a single copy in each of the analyzed strains. A *Nocardia* strain (not shown) was used as an out-group to root the tree.

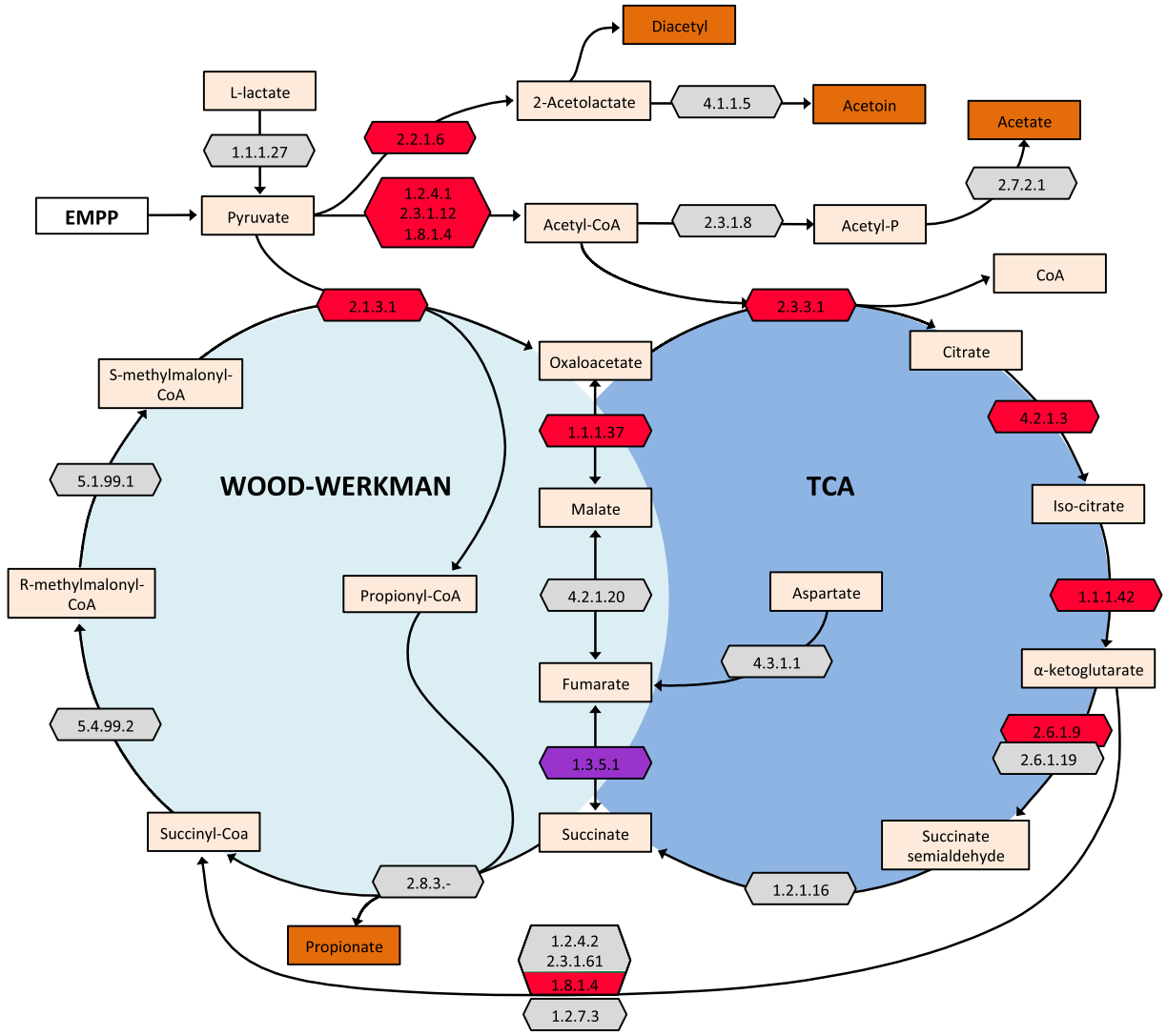

Fig. 5. Schematic representation of selected metabolic pathways in *P. freudenreichii* ssp. *shermanii* JS. Included are Wood-Werkman cycle, citric acid cycle (TCA), and various other flavor formation-related metabolic conversions. Metabolites are given in the boxes and central flavor compounds are highlighted in orange. EC numbers describing the enzymatic reactions are given in hexagons and significant changes in the expression of associated genes are indicated by color. Reactions associated with genes up-regulated in the warm room ripening are designed by red color and reactions associated with genes both up- and down-regulated in the warm room ripening are designated in purple. Grey designates reactions that are associated with genes without considerable expression changes between the two conditions. EMPP stands for Embden-Meyerhof-Parnas-pathway.

butanediol, respectively. Rerouting pyruvate to the diacetyl/acetoin pathway has been suggested to be advantageous for lactococci during sugar fermentation at low pH as this can prevent accumulation of excess pyruvate and subsequent intracellular acidification (Passerini et al., 2013; Zuljan et al., 2014). Similarly, the diacetyl/acetoin pathway annotated for strain JS could be beneficial under conditions promoting excess pyruvate production, such as co-metabolism of aspartate and lactate. When *P. freudenreichii* ferments both aspartate and lactate, the metabolism of aspartate to succinate *via* fumarate drives the fermentation of pyruvate generated from lactate to acetate and CO_2, and these end products are produced more than the end product propionate (Crow, 1986a). The acetoin/diacetyl pathway could therefore serve as an additional route for pyruvate generated in the co-metabolism of aspartate and lactate, and *P. freudenreichii* could thus convert some of the pyruvate into acetolactate and further into diacetyl and acetoin in addition to the known generation of acetate. Moreover, the ability to produce acetoin and diacetyl could be particularly advantageous to strain JS, as the strain demonstrates particularly high aspartase activity (Blasco et al., 2011).

Typical for dairy propionibacteria (Crow, 1986b; Piveteau, 1999), strain JS seemed to rely on the fermentation of L-lactate instead of D-lactate. The genome analysis revealed only L-lactate (*PFREUDJS_001689*), but no D-lactate permease-encoding gene for lactate import. As the previously sequenced *P. freudenreichii* genomes (CIRM-BIA1 and ITG P20) have also only L-lactate permease-encoding gene, the lack of D-lactate permease could be common in *P. freudenreichii*. For the conversion of lactate into pyruvate, strain JS was predicted to harbor only two L-lactate dehydrogenase (EC 1.1.1.27) -encoding genes, whereas the genomes of strains ITG P20 and CIRM-BIA1 contained both D- and L-lactate dehydrogenase genes. Of the two L-lactate dehydrogenase genes of strain JS, one (*PFREUDJS_001231*) belonged to the conserved core, whereas the other (*PFREUDJS_001109*) was variably detected in the analyzed strains, having counterparts in all 20 *P. acnes*, *P. avidum*, *P. freudenreichii*, and *P. humerusii* strains but not in any of the remaining nine strains (Supplemental file 1).

Propionibacteria contribute to the properties of cheese also through lipolysis of milk fat to free fatty acids and catabolism of amino acids to branched-chain acids (Poonam et al., 2012; Thierry et al., 2011). The annotation of the JS genome sequence identified altogether 12 CDSs

belonging to the esterase-lipase superfamily (cl21494; Supplemental file 2), of which one (*PFREUDJS_002089*) matched the secreted esterase PF#279 shown to be active on milk triglycerides and suggested to be the main lipolytic enzyme of *P. freudenreichii* in cheese (Abeijon Mukdsi et al., 2014; Dherbecourt et al., 2010) and one (*PFREUDJS_000145*) matched the previously characterized esterase A that has been shown to have a preference for short chain fatty acids (Suoniemi and Tynkkynen, 2002). Interestingly, ortholog grouping revealed PF#279 to be present in all the *P. freudenreichii* strains and absent in all the other propionibacteria analyzed, whereas esterase A was absent in the other *P. freudenreichii* strains and variably detected in the other propionibacteria (Supplemental file 1). In addition, a counterpart *(PFREUDJS_002100*) for the previously characterized cell wall-anchored esterase PF#774, which is active on milk triglycerides (Abeijon Mukdsi et al., 2014; Dherbecourt et al., 2010), was identified in the genome of strain JS. This enzyme was conserved in all the other *Propionibacterium* species analyzed except for *P. acidifaciens* and *P. propionicum* (Supplemental file 1). Taken together, these results suggest strain JS to have both species-specific and strain-dependent lipolytic characteristics.

The genome of strain JS was also annotated to code for several putative proteases and peptidases as well as enzymes for the degradation of amino acids, including alanine dehydrogenase (*PFREUDJS_000359*), serine dehydratase (*PFREUDJS_001681*), and threonine dehydratase (*PFREUDJS_002140*), the latter of which also catabolizes serine. Two copies of aspartate ammonia lyase-encoding genes (*PFREUDJS_001554* and *PFREUDJS_001551*) for the utilization of aspartate were also identified (Fig. 5). This feature is of industrial relevance as co-metabolism of aspartate with lactate increases the production of acetate and CO_2 at the expense of propionate production and can affect the characteristics of cheese (Crow, 1986a). The JS genome was also predicted to encode aminotransferases involved in the transamination and degradation of amino acids, such a branched-chain amino acid aminotransferase (*ilvE*; *PFREUDJS_001279*) involved in the formation of short branched-chain fatty acids form leucine, valine, and isoleucine. The *ilvE* gene belonged to the conserved core of the analyzed propionibacteria, while the rest of the aforementioned amino acid catabolism enzymes were variably present in the investigated *Propionibacterium* strains (Supplemental file 1). Of note, the aspartate ammonia lyases were part of the same ortholog group and, unlike the other enzymes listed above, had no counterparts in strain ITG P20.

The ability to ferment lactose or nitrate has in the past been used to distinguish lactose degrading, nitrate negative *P. freudenreichii* ssp. *shermanii* from lactose negative, nitrate degrading *P. freudenreichii* ssp. *freudenreichii* (Thierry et al., 2011). In accordance with this subspecies classification, strain JS had genes for the transport and utilization of lactose and lacked some genes reported (Loux et al., 2015) to be required for nitrate reduction. Similar to other lactose degrading *P. freudenreichii* strains (Loux et al., 2015), the lactose utilization genes *lacZ* (*PFREUDJS_002190*), *galE* (*PFREUDJS_002192*), and the transporter-encoding gene (*PFREUDJS_002191*) were embedded in a GI. In addition, strain JS had an additional copy of *lacZ* (*PFREUDJS_001782*) and *galE* (*PFREUDJS_000171*). The two *galE* copies were inparalogs and all the lactose utilization genes of strain JS had counterparts in strains CIRM-BIA1 and ITG P20 (Supplemental file 1), which have both been reported to degrade lactose (Loux et al., 2015).

Some propionibacteria are good producers of vitamin B12, offering an alluring possibility for *in situ* vitamin enrichment of fermented foods (Poonam et al., 2012). Moreover, B12 is required as a co-enzyme by the Methylmalonyl-CoA mutase (EC 5.4.99.2; *PFREUDJS_000662-PFREUDJS_000663*) of the Wood-Werkman cycle (Fig. 5). A complete pathway for vitamin B12 synthesis has been previously described in *P. freudenreichii* ssp. *shermanii* CIRM-BIA1 (Falentin et al., 2010a). Based on our ortholog analysis, the same pathway was also present in strains JS and ITG P20. A majority of the JS genes orthologous to the B12 synthesis genes in CIRM-BIA1 followed the organization of the B12 gene clusters in CIRM-BIA1 (Falentin et al., 2010a), supporting the intactness of these loci in the JS genome.

3.4. Gene expression during an industrial cheese ripening process

Here, a genome-wide transcriptome profiling was used to determine the expression of *P. freudenreichii* ssp. *shermanii* JS genes during warm and cold room ripening of cheese (Fig. 1). Of the ~152 and ~154 million RNA-seq reads obtained from the warm and cold room samples, ~81.2% and ~81.1% passed the filtering process, ~0.3% and ~2.1% mapped to the JS genome, and ~0.2% and ~1.8% overlapped a CDS of strain JS, respectively. The comparison of the RNA-seq and 16S rRNA gene counts using library size-normalized count data revealed a relatively high correlation between feature-mapped RNA-seq reads and *P. freudenreichii* ssp. *shermanii* assigned amplicon reads ($R^2 = 0.65$; p-value ≤ 0.06; Fig. S2). These results are in line with the low inoculum size of *P. freudenreichii* in Swiss-type cheeses, which typically is only few hundred colony forming units per 1000 l of milk (Frohlich-Wyder and Bachmann, 2004), and provided a strong evidence that differences in feature mapped RNA-read counts originated from varying concentrations of bacterial cells present in the samples rather than from the differential expression of genes of a constant amount of cells, establishing thus the validity of TMM normalization (Robinson and Oshlack, 2010) in the RNA-seq data analysis. The bacterial taxa detected in the cheese samples by amplicon sequencing (Table S3) are discussed in more detail in Supplemental text.

A total of 348 CDSs were considered differentially expressed (fold-change ≥ 2 and FDR ≤ 0.05) between the two sampling points. Of these CDSs, 128 were up-regulated and 220 down-regulated during warm room ripening (Supplemental file 2). The enrichment analysis of these genes (Fig. 6) indicated that genes more expressed in the warm were enriched in the energy production and conversion (C), and amino acid transport and metabolism (E) categories, suggesting that *P. freudenreichii* grew faster and was metabolically more active during warm room than cold room ripening. These observations are in accordance with the previously reported strong increase of *P. freudenreichii* population during cheese ripening at warm temperatures and down regulation of genes related to energy metabolism and cell machinery in the cold room (Dalmasso et al., 2012; Falentin et al., 2010b). In contrast, cold-induced genes of strain JS were frequent in the replication, recombination and repair (L) category (Fig. 6), and this enrichment was driven by over 40 mobile element and transposon-associated genes. In addition, a considerable amount of the cold-induced genes lacked a functional annotation. Interestingly, this group included the majority of the differentially expressed strain-specific genes of strain JS.

KEGG pathway category analysis using KAAS server (Moriya et al., 2007) and hypergeometric distribution revealed that 11 out of 81 metabolic pathways with five or more genes were significantly enriched with differentially expressed genes; ten pathways were enriched in genes induced during warm room ripening, whereas only an arginine and proline metabolism pathway was enriched in genes up-regulated during cold room ripening (Supplemental file 2). Pathways enriched with genes over-expressed in the warm room included several large-scale, global KEGG pathways, such as “metabolic pathways” and “carbon metabolism”, supporting the active growth and metabolism during warm room ripening. To further investigate enzymatic alterations between the cheese ripening phases, the up- and down-regulation status of the differentially expressed JS genes was projected on the reference pathways of strain CIRM-BIA1 using the KEGG mapper (Kanehisa et al., 2012). The gene expression colored KEGG maps (Supplemental file 3) as well as the transcriptome data (Supplemental file 2), further demonstrated strain JS to have a more active fermentative metabolism during warm room than cold room ripening of cheese. For instance, several enzymes part of Wood-Werkman cycle and TCA were repressed in the cold room. These changes along with the expression changes of other

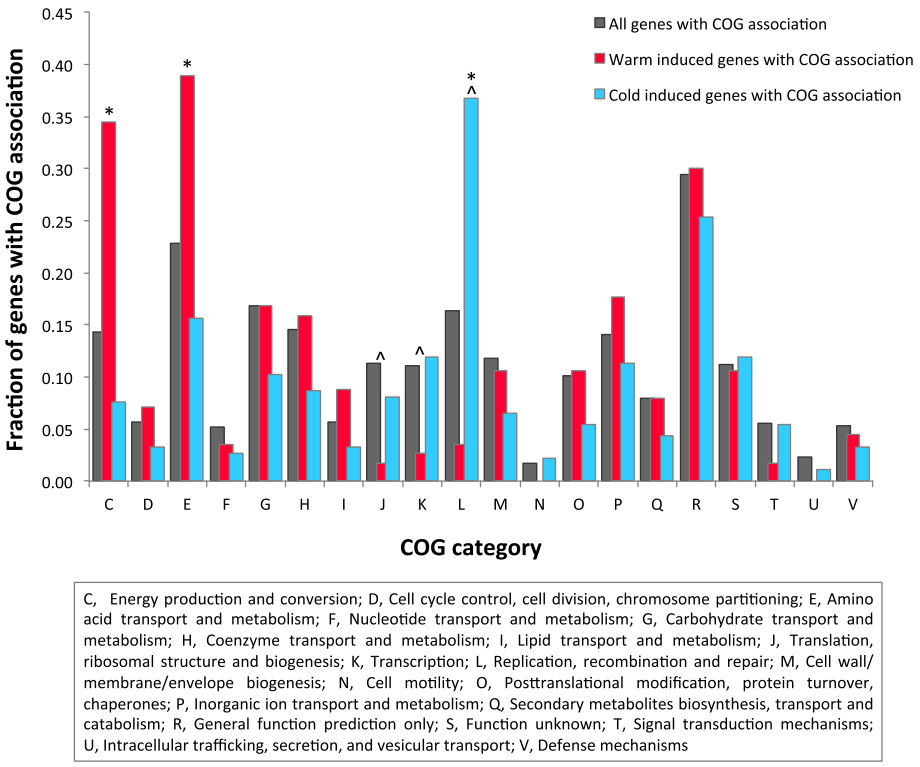

Fig. 6. COG category enrichment analysis. The grey bars represent the fraction of CDSs of strain JS within the given COG categories. Red and blue bars represent the fraction of warm- and cold-induced genes, respectively, of CDSs associated with the given COG category over all warm- or cold-induced genes with a COG association. The significance of the enrichment was assessed using hypergeometric distributions followed by Benjamini-Hochberg correction procedure. * designates categories with significant enrichment and ^ categories with significant under-representation.

genes of relevance to cheese manufacturing are discussed in detail below.

3.5. Expression of flavor-related genes during industrial cheese ripening

The exponential growth of *P. freudenreichii* takes place in warm room (Falentin et al., 2010b), resulting in extensive formation of CO_2 and, consequently, to the development of the characteristics eyes of the Swiss-type cheeses. A majority of the propionate is also produced during warm room ripening (Thierry et al., 2005). Accordingly, the transcriptome analysis performed here revealed several genes of the Wood-Werkman and TCA cycles to have elevated expression levels (fold-change ≥ 2 and FDR ≤ 0.05) in warm room (Fig. 5, Supplemental file 2), suggesting a more prominent production of propionate in warm room than cold room ripening. As the propionate production is coupled with the oxidation of pyruvate to acetate (Piveteau, 1999), it is not surprising that expression levels of some of the genes (*PFREUDJS_000906*, *PFREUDJS_000132*, and *PFREUDJS_001054*) responsible for oxidative decarboxylation of pyruvate were elevated in warm room (Supplemental file 2). The transcriptome profiling also indicated that strain JS produced flavor compounds acetoin and diacetyl from pyruvate especially during warm room ripening (Fig. 5). Specifically, the gene expression data indicated that both acetolactate synthase subunits (EC 2.2.1.6; *PFREUDJS_001285* and *PFREUDJS_001286*), forming the first step in the diacetyl/acetoin pathway, were more expressed in warm than cold room (Supplemental file 2). The acetolactate decarboxylase (EC 4.1.1.5; *PFREUDJS_001113*), catalyzing the acetoin production from acetolactate, was active across the ripening phases (Supplemental file 2), which might be an indication of non-enzymatic conversion of acetolactate to diacetyl (Fig. 5). Interestingly, search for potential regulative motifs over-represented upstream of either warm room ripening or cold room ripening induced TUs (discussed in Supplemental text; Tables S4 and S5) suggested that the genes for acetolactate synthase and methylmalonyl-CoA carboxytransferase (EC:2.1.3.1; *PFREUDJS_001706-PFREUDJS_001709*) of the Wood-Werkman cycle may be regulated by the same regulatory elements. Consequently, the diacetyl/acetoin pathway could be activated under the same conditions as the Wood-Werkman cycle.

Although more active in warm conditions, *P. freudenreichii* displays metabolic and flavor formation activities also at cold temperatures (Dalmasso et al., 2012; Falentin et al., 2010b; Thierry et al., 2005). Similarly, our transcriptome analysis provided clues that strain JS continues to contribute to the development of flavor and other cheese characteristics also in cold room ripening. For instance, the esterase A-encoding gene of strain JS was relatively highly expressed under both conditions (Supplemental file 2), indicating that degradation of fatty acid-esters in cheese continues in cold. The lipolytic esterases PF#279 and PF#774 showed also signs of expression in both conditions, although their expression were rather weak and not detectable systematically across biological replicates (Supplemental file 2). Corroborating the previously reported constant expression of *ilvE* in both warm and cold conditions (Dalmasso et al., 2012), our transcriptome analysis suggested this branched-chain amino acid aminotransferase to retain its activity. These results suggest *P. freudenreichii* to enhance the cheesy/sweaty note of cheese during cold room ripening. Moreover, in an agreement with these expression data, the production of short branched-chain acids in cheese has been shown to occur mainly during cold storage (Thierry et al., 2005).

Majority of the lactose utilization genes of strain JS retained their expression levels across sampling points (Supplemental file 2), which may relate to the low overall utilization of lactose by dairy propionibacteria in cheese. Although the ability to utilize lactose has been suggested to be a characteristic feature of *P. freudenreichii* ssp. *shermanii* (Patrick and McDowell, 2012), the lactose in cheese is primarily consumed by LAB during the very first days of cheese ripening (McSweeney and Fox, 2004) and thus before our first sampling point, at day 12 (Fig. 1).

Moreover, lactate has been reported to be the preferred carbon source by *P. freudenreichii* even in the presence of lactose and some other carbohydrates (Piveteau, 1999).

As described above, the metabolic activity of strain JS was suppressed during cold room ripening, indicating that strain JS was in a stationary phase of growth. Alternatively, the down-regulation of some of the genes coding for components of the Wood-Werkman and TCA cycles in cold room (Fig. 5) might reflect the reduction in lactate concentration during the ripening process (McSweeney and Fox, 2004). The transcriptome data also supported a large-scale transcriptome reprogramming of amino-acid metabolism-related genes between the ripening phases (Fig. 6 and Supplemental files 2 and 3). For example, genes responsible for glutamate-glutamine interconversions (*PFREUDJS_001516* and *PFREUDJS_001140*) were down-regulated in cold, whereas the glutamate decarboxylase gene (*PFREUDJS_002297*) involved in glutamate catabolism was induced. In accordance with the previously observed up-regulation of genes involved in the conversion of serine and alanine into pyruvate at +4 °C (Dalmasso et al., 2012), strain JS up-regulated its alanine dehydrogenase- (*PFREUDJS_000359*) and threonine dehydratase (*PFREUDJS_002140*) -encoding genes in cold room. In contrast, serine dehydratase (*PFREUDJS_001681*) was not differentially expressed and the aspartate ammonia lyase (*PFREUDJS_001554* and *PFREUDJS_001551*) was not repressed but demonstrated borderline up-regulation (Supplemental file 2, Q-value ≤ 0.002 and fold-change ≤ 1.77) in cold room, although the opposite has been previously observed for strain CIRM-BIA1 in laboratory conditions (Dalmasso et al., 2012). In addition to the borderline up-regulation of the aspartate ammonia lyase-encoding genes, a more profound up-regulation of another gene (*PFREUDJS_000075*, EC 6.3.4.5), involved in aspartate utilization and subsequent production of fumarate, was observed in the cold. Interestingly, the expression patterns of these aspartate catabolism related genes did not follow those of the differentially expressed acetate and acetoin/diacetyl pathway genes (Fig. 5), although the co-metabolism of aspartate and lactate could lead to the production of acetate, diacetyl, and acetoin. These results might reflect possible differences in the availability or aspartate and lactate, particularly the exhaustion of lactate during the cheese ripening (McSweeney and Fox, 2004). Nevertheless, the transcriptome data reported here revealed marked amino acid catabolism activity also during cold room ripening. If an expression level estimate of a gene can be used as a proxy for its enzymatic activity, these results provide evidence that *P. freudenreichii* ssp. *shermanii* JS contributes to the properties of cheese in cold room ripening primarily through amino acid catabolism.

4. Conclusions

By combining genome- and transcriptome-sequencing, this study advances our knowledge of the functional genomics of dairy propionibacteria and provides valuable insights into the role of *P. freudenreichii* in cheese ripening. Genome analysis of strain JS revealed systems for the defense against phages (see Supplemental text for details), a known threat to the dairy industry, and pathways responsible for the production of flavor-compounds. Majority of the features identified in strain JS were conserved in other *P. freudenreichii* strains investigated, a finding that is in agreement with the high level of sequence homology observed between the strains. Based on the transcriptome profiling, the central carbon metabolism and propionate production of *P. freudenreichii* were more active during warm room than cold room ripening of cheese. On the other hand, evidence of amino acid catabolism was seen in both sampling points. Taken together, these results indicate that activity of *P. freudenreichii* ssp. *shermanii* JS during warm room ripening drives the development of flavor, although marked activity was also seen during cold room ripening.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2016.09.022.

Conflict of interest

TA, JT, SP, TS, and ST are employed by Valio Ltd and MK, LP, and PA have received funds from Valio Ltd, which produces and utilizes strain JS.

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